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TITLE: A Novel Knock-out Animal Model to Analyze Transcriptional Signaling by p53 Tumor Suppressor Protein in Breast Cancer

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13. ABSTRACT *(Maximum 200 Words)*
Two important transcriptional targets of p53 tumor suppressor protein are genes encoding the Proliferatng Cell Nuclear Antigen (PCNA) and p21/WAF1/Cip1. PCNA is a necessary component of DNA replication and DNA repair machinery. p21/WAF1/Cip1 is a cyclin-dependent kinase (cdk) inhibitor which can interact with PCNA to modulate the balance of DNA repair versus replication. We hypothesize that correct ratio of PCNA and p21 proteins is crucial for normal reglation of DNA repair and cell cycle control, and hence, disregulation of pcna and p21 transcription in response to genomic damage is an important aspect of breast cancer formation. To test this hypothesis in vivo, we are developing a mouse model where p53 signaling *specifically* to the pcna and p21 gene transcription is disrupted. Toward this goal, we are characterizing p53 interaction sites on mouse p21 and pcna gene promoters (from a series of BAC clones isolted with the help of Roswell Park Cancer Institute Microarray and Genomics Facility). This mouse model will enble testing the relevance of specific transcriptional signaling by p53 to mammary oncogenesis, identifification of new therapeutic targets, and analyzing the role of specific p53 transcriptional targets in modulating responses to chemotherapeutic drugs and radiation therapy.

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INTROUCTION:

Elimination of functional p53 tumor suppressor protein through various mechanisms is the single most common event in human cancer, occurring in more than 50% of human cancers (1). p53 elicits its biological effects mainly by functioning as a transcriptional regulator of various target genes (2). Two such p53 targets important in cellular response to genomic damages are genes encoding the Proliferating Cell Nuclear Antigen (PCNA) and p21/WAF1/Cip1 (3). PCNA is a necessary component of DNA replication and DNA repair machinery. It forms a doughnut-like structure around DNA thereby forming a sliding platform for interaction of various proteins involved in DNA repair, DNA replication, and cell cycle control. p21/WAF1/Cip1 is a cyclin-dependent kinase (cdk) inhibitor which can interact with PCNA to modulate the balance of DNA repair versus replication.

In response to genomic damage, p21/WAF1/Cip1 is induced at the transcriptional level by p53. Results from several laboratories including ours have shown that PCNA is co-induced with p53 in response to genomic damage, and that p53 is a transcriptional activator of PCNA (4). However, the mechanisms by which p53 exert transcriptional regulation of PCNA and p21/WAF1/Cip1 genes in normal cells and in cells subjected to genomic damage remain unknown. We hypothesize that correct balance of PCNA and p21 amounts is crucial for normal regulation of DNA repair and cell cycle control, hence, dysregulation of PCNA and p21 transcription in response to genomic damage is an important aspect of breast cancer formation. To test this hypothesis in vivo, we are developing a mouse model where p53 signaling specifically to PCNA and p21 gene transcription is disrupted by knocking out the p53 interaction sites on the promoters of the genes. Since we will be disrupting p53 signaling to the PCNA or p21 transcription ONLY at a time, this model will facilitate analyzing the role of gene-specific transcriptional regulation by p53 in breast cancer. Such analysis has been impossible with the conventional p53 knockout (p53-/-) mouse model where regulations of multiple p53 target genes besides PCNA are disrupted. This animal model will enable us to analyze in breast tissue the developmental and physiological consequences of uncontrolled PCNA and p21 expression when their transcription is divorced from the regulatory role of p53. Moreover, unlike conventional knockouts, our model will not be completely compromised for PCNA expression, thereby preventing the possibility of embryonic lethality. PCNA is expressed in cells, which are null for p53, and these cells can divide suggesting that they are capable of DNA replication. We hypothesize that is DNA repair that would mainly be affected if PCNA and/or p21 transcription is unlinked from p53 control, and hence, more relevant for tumorigenesis.

The novel mouse model proposed here will provide physiological in vivo experimental system to test the relevance of specific transcriptional signaling by p53 to mammary oncogenesis, which in turn, could be used for identifying specific signaling pathway targets in developing therapeutic strategies to combat breast cancer. This

would also be a promising system to analyze the role of specific p53 transcriptional targets in modulating responses to chemotherapeutic drugs and radiation therapy.

BODY:

The first step toward generating the mouse model described above was to characterize the mouse pcna gene promoter with respect to transcriptional regulation by p53. Although the p53-binding site on the human pcna gene is well documented (including work from our laboratory), there are no reports available on p53 sites on the mouse pcna gene promoter. Also, relatively short region of the mouse gene promoter has been reported thus far. Therefore, our first attempt was to obtain long stretches of mouse pcna promoter. We wanted to obtain the gene from the same strain of mouse that is the source of embryonic stem cells (ES cells) to be used for making the chimeric mouse. Introduction of the altered pcna and p21 genes into ES cells, generation of chimeric mouse, and finally mice homozygous for the pcna and p21mutant genes will be accomplised with the help of Gene Targeting and Transgenic Facility at the Rosewel Park Cancer Institute, Buffalo, New York. The ES cells used at this facility was derived from 129SVJ strain of mouse. Therefore, we decided to screen a bacterial artificial chromosome BAC) library (RPCI-22) of genomiic DNA from 129SVJ mouse strain for genomic clones of pcna and p21 genes.

Screening of BAC library:

The screening of BAC library was performed with the help of the DNA Microarray Facility Facility at the Rosewel Park Cancer Institute. Sequence of mouse p21pcna genes was obtained from Celera mouse database. DNA sequences identified from the database and the sequence of the 40 bp overgo probe for screening the RPCI22 IBAC library are shown below:

>p21_5_UTR (120-200 specific)
aagaccagagggagcctgaagactgtgatggggtagtttccatagtgacccgggtccttcttgtgtttcagccacagcgac
c

Complete overgo sequence for P21_5_UTR2 :
AGCCTGAAGACTGTGATGGGGTAGTTTCCATAGTGACCCG

>pcna_5_UTR (highly specific)
taggggtgttaaaatatggtggcctctttattactcgatattttgcagcgtatttcttacgttagggaaaacgctccgtagtgttta
aaatactctccagcttcaaggcaggccgccgcgcacagctcgatttgcctgtgacttccacttccgtggcgcggaaacttcc
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gcctcccaggctcctaccccgcagccccgcctttgcatacgcggtggggcgggccttgctcaaaccacgggtacgattgg
tccttgaggagaggtgggtggatcagcgctgtggcgtcatgacctcgcgcagggaaaaggcgcgcgcctaggaagccg
cg

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Complete overgo sequence for PCNA_5_UTR :
CGCGGAAACTTCCTAAGGATGGAAACTGCAGCCTAAACTC

Initial screen of the BAC library was performed by Southern hybridization using the overgos specific for p21 and pcna as probes. 18 positive clones were obtained. To confirm the authenticity of the clones, all the 18 clones were re-screened by PCR with oligonucleotides specific for p21 and pcna genes. From the PCR screen, 14 clones were confirmed positive of which 11 were p21 clones and 3 were pcna clones.

Identification of p53-binding sites on pcna gene promoter.

For disrupting the p53 signaling specifically to the pcna and to the p21 gene trnscription, it is essential to know what are the p53-responsive sites on the proter regions of these genes. As discussed earlier, p53-responsive elements are reasonably well characterized in the case of p21 gene, whereas no such information exists for the mouse pcna gene. As a first step toward unraveling the p53 –responsive elements on the pcna gene promoter, we searched pcna promoter region (3496 bp upstream of the transcription initiation site) for consensus p53-binding site. The results we obtained in a search using the MacVector software are as follows:

```
Sequence Range: 1 to 3495

         10        20        30        40        50        60
CTTGCTCTCCCTGTGGTCCTGAGTTCCAATCCTAACAACCACATGGTGGCTCACAACCAC
GAACGAGAGGGACACCAGGACTCAAGGTTAGGATTGTTGGTGTACCACCGAGTGTTGGTG

         70        80        90       100       110       120
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GGTTTTACTCTAGACTACGGGAGAAGACCACATGTCGATGTCACATGAATACATATTATT

        130       140       150       160       170       180
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ATTCATGTAGAAATTTTATTATTATTATTATTATTATTATTATTATTTTATTTTATA

        190       200       210       220       230       240
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GAGGTCCTCCGACTCCGTATCGAGTTTCCGCCCAAAGACAAATCGAAAAGACTTCAGGAC

        250       260       270       280       290       300
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TGTAGTCAAGGGTCGTAATTCTTTTTCAGAGATTTTATTAATGTAAAGATCCGTAATTTC

        310       320       330       340       350       360
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TTTTATAATATATTCGGGGGTCGTGGCTCTGTCTCGTTGTCTTCTTTGTATCACAACAAT

           >p53
             |
        370  |    380       390       400       410       420
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CACAACAATCGTTTCGTTCTGGTGTTCAGTCTTTCTTGTGTAAGATACCAAACGTTTCCT
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       430        440        450        460        470        480
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TTACTCTAAAATTCTTTGAGTTCTTTTCTTTTAAGAGTGTTTATATACATCGACATACTA

       490        500        510        520        530        540
GGGTTTTTATTTTTGTTTGTGCTGTGTTGTTGGACACACAGTAACACTAAGCTGCAAGAC
CCCAAAAAATAAAAACAAACACGACACAACAACCTGTGTGTCATTGTGATTCGACGTTCTG

       550        560        570        580        590        600
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GTCAGGATGTAATAGAGAACGACACACTTCCTTATTAAGTCCGACAGTTCTGTCGAATCG

       610        620        630        640        650        660
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       670        680        690        700        710        720
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     <p53
      |
      |730        740        750        760        770        780
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       790        800        810        820        830        840
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                                    >p53      <p53
                                     |         |
       850        860        870    |   880   |    890        900
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       910        920        930        940        950        960
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       970        980        990       1000       1010       1020
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CTATCGTGATGATATTTGTCTGTCACACTTGTGGAGAGACAGCACAGAAGACACTCCTAC

                                  <p53
                                   |
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CTCTAGTACACAACATGTCCAGTCCGTTCGTGAGATAGTGATACATTGGGATCGAGGGTA

      1090       1100       1110       1120       1130       1140
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                                                          <p53
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                                                                    |
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GTGCGGGCCGAGGGTACAAAAAGAATTTTCTATGGGTGTTCCCATTATATACTAATTCCC

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CACCCCCGACTTTCATATCATCACCCCAAAAGACCCAAAACAAAAATTAAAATTTTTTTA

      1390       1400       1410       1420       1430       1440
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                                                          <p53

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                 |
                <p53
                 |
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      2050       2060       2070       2080       2090       2100
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                                              <p53
                                               |
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      2170       2180       2190       2200       2210       2220
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      2230       2240       2250       2260       2270       2280
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                                                    <p53
                                                     |
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      2350       2360       2370       2380       2390       2400
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                >p53
                 |
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      2830       2840       2850       2860       2870       2880
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      2890       2900       2910       2920       2930       2940
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      2950       2960       2970       2980       2990       3000
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                                                 <p53
                                                  |
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      3490
CCTAGGAAGCCGCGG
GGATCCTTCGGCGCC
```

Further sequence examination in terms of conservation of crucial residues suggested that sites at 727, 1052, and at 1555 are the most likely functional sites.

It is essential to ensure the functional relevance of the binding sites identified based on sequence homology. To address this issue, we are planning to verify which one of these sites is transcriptionally p53-responsive in a transfection assay. Toward this goal, we are in the process of cloning the > 3 kb pcna promoter fragment into pGL3 Luciferase assay vector that has been extensively used in our laboratory for transcription assays in vivo.

Once we ascertain the functional authenticity of p53 sites on the mouse pcna and p21 gene promoters in a highly sensitive luciferase reporter-based transcription assay, we will also construct mutant versions of the promoter where the p53-binding site(s) are functionally debilitated by mutagenizing critical residues. We will clone a large region (~ 9kb) of pcna gene promoter encompassing the mutant p53-binding sites into a ES cell targetting vector. The ES cells carrying these mutant pcna gene will be injected into blastocyts to create chimeric mouse followed by breeding to generate mice which are homozygous for the engineered mutation. To direct conditional p53 signaling disruption to the pcna gene in mammary tissue, we will use the Cre/lox-recombinase system (5). Using a similar strategy, we will also generate mice that are homozygous for mutant p21 gene.

KEY RESEARCH ACCOMPLISHMENTS:

- Isolated mouse genomic clones of pcna and p21 genes.
- Identified putative p53-interaction sites on the pcna gene promoter.

REPORTABLE OUTCOMES:

Too early to be submittted as a manuscript for publication.
An abstract has been submitted to the upcoming third Era of Hope meeting to be held in Florida, September 25-28, 2002.

LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:

Hui Yang, M.D., Ph.D.
Ying Yang, M.S.

CONCLUSIONS:

Once we succeed in generating the mouse carrying mutations that would specifically disrupt p53 signaling to the transcriptional regulatory machinery of pcna and p21 genes, such mice will be an important model system to analyze mechanisms of mammary oncogenesis.

"So What?"

Information gained could be used for identifying specific signaling pathway targets in developing therapeutic strategies to combat breast cancer. This would also be a promising system to analyze the role of specific p53 transcriptional targets in modulating responses to breast cancer chemotherapeutic drugs and radiation therapy.

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DEPARTMENT OF THE ARMY
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